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I. Work Summary

A. Purification of LPS binding factors in tolerant serum by affinity chromatography

Since the last report, we have completed another two experiments using this approach. In each, large quantities of LPS were preincubated in normal or tolerant (inflammatory) rabbit sera and then diluted 1:1 with PBS and passed over a previously prepared affinity column consisting of a murine monoclonal IgG specific for the O-polysaccharide antigen of the LPS coupled to Separose 4B. The column was then extensively washed and the bound complexes eluted with 0.1 M glycine adjusted to pH 2.8 and then analyzed by SDS-PAGE. In prior experiments we had problems with non-specific binding to the column, and in addition we were unsure that we were working with enough material to detect the LPS binding factor(s) which we are seeking to isolate. Accordingly, in these two experiments we scaled up the column and the quantity of LPS and in addition we added 150 mM NaCl to the buffers in an attempt to decrease non-specific binding. We also step-eluted the bound complexes in the hopes of eluting non-specific proteins separately. Another problem we had in the past was the need to desalt and then concentrate the relatively large volume eluted (about 20 ml) to very small volumes (about 50 ul) for analysis without losing substance to the walls and filter. Our most recent attempt consists of a very rapid dialysis followed by lyophilization. Despite all of these changes, in each of the experiments we had several protein bands which were identical in both the normal and tolerant serum experiments. We interpret these results either to mean that:

a. We have not yet solved the non-specific binding problem.

b. That there are substances in normal serum which bind to LPS and in fact the repeated finding of protein bands in normal serum represents a real and potentially important finding.

We plan to address these issues in three ways. First, we will utilize controls with a heterologous LPS preincubated in both normal and tolerant serum on the same column. This LPS should not be captured by the column. If the bands are still present, this will constitute evidence that the bands we see are non-specific and that we should focus our energy on methods to further non-specific protein binding (i.e. with higher salt concentrations and/or the use of non-denaturing detergents). If the bands disappear, this will constitute evidence that the bands in the normal serum (as well as the tolerant serum) may be real. Second, since the SDS in the gels should disrupt the complexes, we plan to try blotting out gels onto nitrocellulose paper, then incubating with a heterlogous LPS, and developing the paper as if it were a Western blot using an anti-LPS antisera and appropriate staining techniques. We have had good results with this technique for demonstrating the binding of other proteins to LPS. (For

this experiment we will use an LPS binding protein from Limulus Lysate as a positive control, which works well in our hands.) Third, we will use tritiated LPS at all phases of the experiments to be sure that we can account for the location of the LPS and its potential loss by adsorption to the surfaces of our tubes and filters.

Towards the end of the last period we radiolabeled E. coli O111 in the LPS component using the gal E mutant of this strain in media containing tritiated galactose. We then used the hot phenol method to extract the radiolabeled LPS. Our yield was 2.53 mg of LPS with a specific activity of 15800 cpm/mg. We will use this reagent in the above experiments.

B. Purification of LPS using a repetitive protein purification approach.

We have previously found that radiolabeled LPS is precipitated from tolerant serum to a much greater extent than from normal serum with calcium and dextran, and this forms the basis for a radioimmunoassay to isolate the responsible factor(s). We found that lipoproteins purified by ultracentrifugation from tolerant sera, but not normal sera, contain activity. Since the last report we have isolated the lipoproteins from 10 mls of normal and tolerant sera by ultracentrifugation and prepared frozen 1.0 ml aliquots which contain activity. We passed these over a 1 meter sepharose 6B column and also over a Superose 6 HPLC column. We found that while the elution profiles of the lipoproteins from normal and tolerant sera were different, that activity in our radioimmunoassay was lost in passage over each column. As in the experiments described above, we believe that a difficulty with the large column is that we may be losing activity in the process of concentrating the fractions. As noted in our original proposal, losses by adsorption to glass and plastic ware are likely to be high because of the hydrophobicity of the lipoproteins. We are now concentrating on the HPLC which has a higher resolution and which permits the repetition of many identical runs. As we have documented that the material going onto the Superose 6 HPLC column has activity and that a concentrated pool of all fractions coming off the column has no activity, it may well be that some or all of the factor(s) may be sticking to the beads. Thus our next step will be to elute the column with a variety of non-denaturing solvents with the hopes that we can recover activity (and thus use the column as a purification step by adsorption). If this approach fails, we plan to turn to columns based on hydrophobic interactions (phenyl or alkyl Superose) and on cation exchange to try to explore the likely hydrophobicity and positive charge of most known LPS binding substances.

C. Related Projects

1. Interactions of LPS in polyclonal serum to rough mutant E. coli J5.

The substances in this serum which bind LPS are of considerable interest because J5 antiserum protects against LPS from all gram negative strains in animal models. Monoclonal antibodies to E. coli J5 have been described which protect, but there is little convincing evidence that these antibodies bind LPS. Thus the interactions of LPS in this antiserum may lead to insights as to how the antiserum protects as well as a means to develop monoclonal antibodies which bind LPS.

We have recently found that the radioimmunoassay described above using calcium and dextran precipitates several tritiated LPS from J5 antiserum but not normal serum. Purified IgG from J5 antiserum but not IgG from normal serum also precipitates tritiated LPS in the assay, although the precipitation is absolutely dependent on the presence of at least small concentrations of normal serum in the reaction mixture. Normal serum containing all the serum proteins, but not the serum lipoproteins will not perform this facilitating function. It thus seems as if the LPS interacts with elements in the normal serum, perhaps exposing epitopes which are then available to bind to the IgG in the J5 antiserum. These findings are in press in J. of Infectious Diseases. In the last several months, we have also found that IgG will immunoprecipitate heterologous tritiated LPS, again only in the presence of small quantities of fresh normal serum which contains lipoproteins. Since the binding of the IgG to the LPS is critically dependent on the interactions of the LPS with normal serum, we believe that further study of this interaction will be worthwhile.

2. Analysis of plasma units from normal blood donors for IgG to the O-polysaccharide antigen of multiple LPS.

IgG to the O-polysaccharide antigen of gram negative bacteria is protective. However, the presence of thousands of different O-antigens has precluded developing a immune globulin for the treatment of gram negative infections. In a pilot project, we screened 202 random fresh frozen plasma units for IgG titers to 15 different O-antigens. Classical theory suggests that the IgG titers to each would be random and would be reflective of their prior exposure to each strain. We found that the range of titers to each O-antigen varied about 100-fold, and that some units (about 5%) contained high titers (top 10%) to most of the antigens. Similarly, some units contained only very low titers (bottom 10%) to most of the antigens. The units with high titers did not correlate with titers to E. coli J5. These findings suggest that natural IgG titers to the O-antigen of LPS are not random. We speculate from this pilot study that the donors with low titers may be at higher risk for sepsis and shock than the average donor, and also that it may be possible to use a screening approach to develop an immune globulin which contains high titers to the LPS from multiple gram negative strains. We plan to write these results up for publication, and hope to append a copy of the article to the annual report in 4 months.

II. New Knowlege

1. Our findings from the immunopurification approach together with the findings concerning the need for normal serum in the binding of LPS to IgG in J5 antiserum suggest that LPS may interact with elements in normal serum as well as those in tolerant serum.

2. LPS binding activity is lost from tolerant serum when passed over sepharose columns. These columns may be helpful as affinity columns.

3. The assays which we developed for the binding of LPS to elements in tolerant sera permit the measurement of the binding of LPS to IgG in antiserum raised to antiserum to rough mutant E. coli J5, which is protective in many assay systems (as well as in human trials).

4. Binding of LPS to IgG from J5 antiserum appears to depend upon an interaction between LPS and an element present in normal serum. Delipidation removes this element.

5. IgG titers to multiple O-antigens of LPS in normal plasma donors appear to be correlated, whereas they were previously felt to be random. Depending upon the magnitude of the effect, this may permit the development of an immune globulin against multiple gram negative strains.

III. Technical Problems

1. In our immunoaffinity columns, we may continue to have problems with non-specific adsorption. These findings may however be real. We need to do further experiments to see if there are LPS interactions in normal serum which account for our results A control with LPS which is heterologous to the IgG on the affinity column should help us resolve the problem.

2. We continue to worry that we may be losing substantial quantities of the hydrophobic factor(s) which we are studying to the walls of the glassware. We are persisting in our attempts to minimize the loss by diminishing the surface area as much as possible. We are also attempting to think of ways around the problem. It may be worth setting aside time to specifically and intensively study the problem by utilizing a number of non-denaturing detergents.

IV. Future Directions

We feel that we are making slow but steady progress towards the characterization and consequent isolation of the LPS binding factor(s) which we are seeking. While we have not yet achieved a final purification, we are gaining in our understanding of the interactions of LPS with elements in serum, and in fact, we believe that we are beginning to see spinoff benefits from our work in related fields.

The finding that the binding of heterologous LPS to IgG in J5 antisera depends on the presence of LPS interacting with something in normal serum which is removed by delipidation may have very important ramifications both in the understanding of the mechanism(s) by which this antiserum protects and also in the development of new monoclonal antibodies which should bind LPS with a much higher affinity than those described. This is an extremely active and rapidly moving area of research. Accordingly, while continuing out outlined projects, we plan to also pursue the interactions of LPS in this antiserum. The reagents and techniques which we have at hand for the study of the binding of LPS to factors in tolerant sera are ideal for this in animal models.

Similarly, our finding that there may be a small percentage of blood donors which contain high titers to multiple LPS O-antigens may permit the development of a protective immune globulin. Accordingly, we plan to compare the protective efficacy of plasma from these donors with plasma which contains only low titers to multiple O-antigens in animal models.

V. Goals for the next trimester
(realizing that it may be optimistic to complete them all within several months)

1. Repeat immunoaffinity experiments using a heterologous LPS as outlined in A. above.

2. Pass purified lipoproteins over Superose 6 HPLC column, assess activity in the radioimmunoassay before and after, and then elute adsorbed material and test for activity. Try non-denaturing detergents. Try hydrophobic columns and cation exchange columns.

3. Radiolabel 8-10 heterologous smooth LPS with tritium as a panel of standard reagents.

4. Compare normal, tolerant, and J5 antiserum using radioimmunoassay and above panel to study binding. Purify IgM from each serum and study using radioimmunoassay both alone and in the presence of fresh normal serum. Study J5 antisera in addition to normal and tolerant serum in the immunoaffinity experiments.

5. Study protective efficacy of screened high titered plasma in actinomycin D sensitized mice.